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Cells have the remarkable ability of detecting specific external stimuli and translating them into growth, differentiation or death. These responses are mediated by signal transduction pathways such as mitogen-activated protein (MAP) kinase pathways conserved from yeast to man. Because of their roles in regulating cell growth, differentiation and death, improper activation of MAP kinase pathways has implicated in breast cancer and other proliferative diseases. The flow of intracellular signaling information is mediated by a series of protein-protein interactions that often take place on scaffold proteins. Scaffold proteins are postulated to tether together a set of pathway proteins and help them act on each other. The flow of signaling information can be artificially modulated to create a novel input-output relationship by engineering scaffold proteins which recruit a unique set of signaling proteins. This strategy of pathway engineering provides for a novel means to treat diseases caused by signaling defects. The progress of breast cancer is determined by a precise balance between growth signaling and death signaling inside cell. One way to treat breast cancer could be the suppression of growth signaling, the promotion of death signaling or both in cancer cells. Goal in this study is to shunt the growth signal to cell death in breast tumor cells and to study in detail the regulatory mechanisms of MAP kinase signaling by identifying negative regulators.

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INTRODUCTION

In human tissues, three major mitogen-activated protein (MAP) kinase pathways exist, ERK, JNK and p38 pathways.[1] These pathways play a critical role in regulating cell proliferation, differentiation and apoptosis, and thus are important targets for therapeutic intervention of proliferative diseases including breast cancer.[2] A hallmark of these pathways is remarkable specificity: a given stimulus leads to a precise response in spite of many related or overlapping pathways within the cell. An emerging paradigm is that such specificity is often mediated by scaffold proteins that physically tether pathway members and restrict them act only on one another.[3] Mechanisms by which MAPK scaffold proteins act on signaling flux are unclear. Recently, it was demonstrated that engineered scaffold proteins in yeast can be used to divert the flow of signaling from one MAP kinase pathway to another, resulting in a non-natural signaling pathway with a novel specificity.[3] Goal in this study is to test the pathway engineering hypothesis as a means to rewire flow of signaling in cells in diseased states. The progress of breast tumor is determined by a delicate balance between proliferation and apoptosis.[4] The proposed study was to divert proliferation signals to apoptotic pathways in breast tumor cells to achieve a simultaneous increase in apoptosis and decrease in proliferation. For signal diversion, upstream components of proliferation pathways (ERK MAPK pathway) is recruited to downstream components of apoptotic pathways (Caspase, p38 and JNK MAPK pathways) via covalent linkage. In addition to the positive regulators of MAPK signaling such as scaffold proteins and kinases, regulatory mechanisms of negative regulators of signaling such as phosphatases are also investigated.

BODY

1. Cloning and expression of PDZ fusion proteins in tumor cells

To redirect the flow of signaling information from proliferation signaling pathway to apoptotic pathway, PDZ fusion proteins were constructed. One PDZ domain was fused to an upstream component of proliferation pathway and another PDZ domain which has an affinity for the first PDZ domain was fused to a downstream component of apoptotic pathway. First, a PDZ domain from syntrophin (synPDZ) was fused to the C-terminus of human Grb2 protein (Shc-Grb2). Second, the PDZ domain from a neuronal nitric oxide synthase (nNOSPDZ) was fused to the C-terminus of the Bad protein (Bad-nNOSPDZ). The PDZ fusion proteins were subcloned in a mammalian expression vector pCDNA3 (Invitrogen). To test the stable expression of PDZ fusion protein in vivo, the fusion constructs were transfected into human breast cancer cells (MCF-7). For the purpose of detection and purification, the fusion proteins were expressed with a six histidine tag (His6) at their N-termini. Expression of PDZ fusion proteins were tested by western blotting using antibodies specific toward the His6 tag.

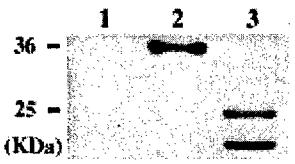

Fig1. anti-His6 immunoblot of Grb2-synPDZ. Lane 1; control tumor cells lacking the expression vector. Lane 2; His6-Grb2-synPDZ expressed from bacteria as a positive control. Lane 3; tumor cells expressing His6-Grb2-synPDZ

For a positive control of immunoblotting, His6-Grb2-synPDZ was expressed from bacteria. The His6-Grb2-synPDZ fusion protein expressed from bacteria was insoluble but detected as a full-length protein (36 kDa). However, His6-Grb2-synPDZ expressed from the tumor cells was expresses as two distinct smaller fragments with the molecular weight of ~ 26 kDa and ~ 20 kDa. It appears that His6-Grb2-synPDZ was proteolyzed inside cell and failed to express as a full-length protein. Fusion of PDZ might have induced a conformational change in Grb2 that destabilized the protein. The anti-His6 immunoblot of His6-Bad-nNOSPDZ showed that the fusion protein was proteolyzed in more significant manner and did fail to express as a full-length protein. (data not shown)

Due to the failure to express the full-length proteins in tumor cells, further study to monitor the rewiring of signaling could not be carried out. However, the failure caused a revision in the direction of the proposed study, in which the hypothesis of signaling control to be tested in a simpler model system, the budding yeast *Saccharomyces cerevisiae*.

2. Regulatory mechanism of MAP kinase signaling pathway

Artificial rewiring of cellular signaling pathways requires detailed knowledge in the regulatory mechanisms of those pathways. One aspect of looking at the regulation of kinase signaling cascades is the balance between positive flux and negative flux. Factors responsible for positive regulation include kinases and scaffold proteins and negative regulatory factors include

phosphatases and proteolysis. In contrast to the vast knowledge and the research efforts toward kinase regulation mechanisms, mechanisms of phosphatases as a key negative regulator remain unclear. It is due to the fact that many phosphatases function in a redundant manner and thus knockouts of phosphatases did not show clear phenotypes in various organisms.

Here, I propose a novel approach to identify phosphatases specific to kinase signaling pathways. The budding yeast *Saccharomyces cerevisiae* was chosen as a model system because it carries a well characterized mating MAP kinase signaling pathway, which is similar in high degree to the mammalian ERK MAP kinase pathway in the structure and organization. The yeast mating MAP kinase pathway consists of a scaffold protein Ste5 and three kinases, Ste11, Ste7 and Fus3, all of which are essential for signaling and possibly are positive regulators. Ste5 is known to interact with all three kinase and provide the platform for signaling to occur. To identify phosphatases specific to mating pathway, all 30 phosphatases in yeast were enforced in proximity to the scaffold complex by a covalent fusion to Ste5 and mating flux of cells carrying each Ste5-phosphatase fusion was monitored. Cells carrying a fusion protein of phosphatase specific to mating pathway are expected to show the decrease in the mating flux.

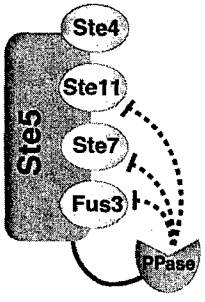

Fig2. Strategy to identify phosphatases that negatively regulate the mating MAP kinase pathway

Change in the mating flux in cells carrying Ste5-phosphatase fusions were monitored by quantitative mating assay. Of all the phosphatase fusions tested, four showed remarkable decrease in the mating efficiency. Of those four, two phosphatases (Msg5 and Ptp3) were known to act on Fus3. However, the other two phosphatases (Pps1 and Ppq1) are previously unknown to participate in the regulation of mating signaling.

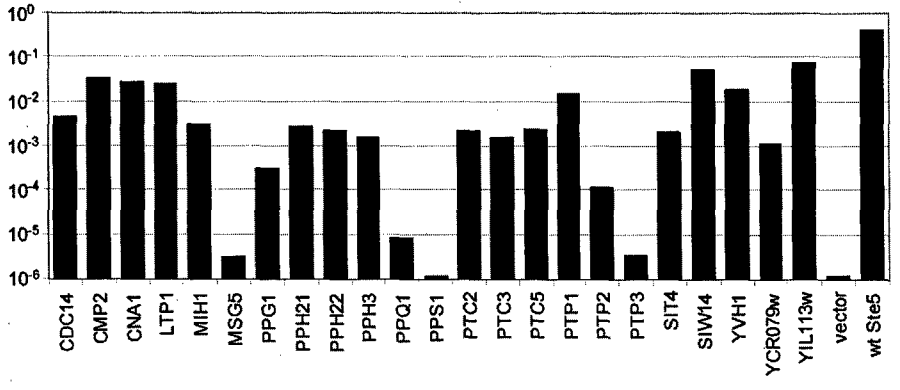

Fig3. Mating test of yeast cells carrying Ste5-phosphatase fusions.

The validity of this approach to identify novel regulators of signaling is proven because the known phosphatases were scored in the screen. Further study is required to investigate the role of the phosphatases (Pps1 and Ppq1) in the regulation of mating signaling, to identify the target substrates of those phosphatases, and to apply this approach in higher eukaryotes.

KEY RESEARCH ACCOMPLISHMENTS

- PDZ fusions of Grb2 and Bad proteins did not express well in the tumor cells.
- A novel strategy to identify negative regulatory factors of MAP kinase pathways was demonstrated.
- Previously unknown phosphatases were identified to function as negative regulators in the mating response MAP kinase pathway in yeast.

REPORTABLE OUTCOME

A manuscript is in preparation based on the findings in this study.

CONCLUSION

The originally proposed study of shunting the signaling from proliferation to apoptosis could not be carried out to its completion because of poor expression of key molecules inside cells. The artificial rewiring of cellular signaling requires high degree of knowledge in the regulatory mechanisms of such pathways. However the difficulty and the lack of detailed knowledge lead to a revised approach to ask what are the key factors in the regulation of cellular signaling and how to identify them. To answer these questions a novel strategy to screen and identify negative regulators of signaling pathway was proposed and tested. The proposed approach was tested in the yeast mating MAP kinase pathway because the yeast model system allows for the high degree of flexibility in genetic manipulation and because the mating MAP kinase pathway is highly homologous to the ERK MAP kinase pathway in mammals in its structure and organization. The screen resulted in the identification of two novel phosphatases that act as negative regulator in the mating signaling as well as known phosphatases previously implicated the pathway. It turns out that the screen is an efficient strategy to identify negative regulatory factors of kinase signaling networks. Further study should be carried out to extend the application of the approach to higher mammals including human.

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